MOLECULAR CHARACTERIZATION OF A NEW *CARLAVIRUS* DETECTED IN GERMAN *ULMUS LEAVIS* (PALL.) BY ILLUMINA HIGH THROUGHPUT SEQUENCING

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SUMMARY

A 150 years old population of 30 European white elms (*Ulmus laevis* Pall.) in the park of Caputh (federal state Brandenburg, Germany) was monitored regularly since 2010. Trees show a dieback and develop characteristic leaf symptoms such as chlorotic ringspots, mottling and necroses. Previous investigations revealed the presence of particles with flexible morphology. To uncover putative pathogenic viral candidates, Illumina high throughput sequencing was applied and three contigs of a putative *Elm carlavirus* were obtained. They show 40.1–74.0 % identity with whole genome sequences of members of the genus *Carlavirus*, family *Betaflexiviridae*. Phylogenetic analysis of whole genome and the replicase polyprotein group them together with *Elderberry carlavirus A*, *B* and *D* and *Poplar mosaic virus* respectively. The analysis of proteins coded within ORF 1-5 show significant characteristics, which are distinctive features for carlaviruses. These results suggest strongly the presence of a so far unknown carlavirus in *Ulmus laevis*.

Key words: Illumina high throughput sequencing, carlavirus detection, *Ulmus leavis*.

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INTRODUCTION

Carlaviruses are widespread within herbaceous plants, but also affect deciduous shrubs and woody plants such as poplar (*Poplar mosaic virus* – PopMV, Smith and Campbell, 2004), *Hydrangea macrophylla* (Hydrangea chlorotic mottle virus - HCMoV, Caballero *et al.*, 2009; Tang *et al.*, 2010), *Vaccinium corymbosum* (*Blueberry scorch virus* – BlSCV; Moretti *et al.*, 2011), *Ligustrum obtusifolium* (*Ligustrum virus A* – LVA; Igori *et al.*, 2016; *Ligustrum necrotic ringspot virus* – LNRSV; Scott and Zimmerman, 2008), *Daphne* spec. (*Daphne virus S* – DVS; Fujita *et al.*, 2015), *Sambucus* spec. (*Elderberry carlavirus A* - EBCVA, *Elderberry carlavirus B* - EBCVB, *Elderberry carlavirus D* - EBCVD, *Elderberry carlavirus E* – EBCVE; Ho *et al.*, 2016) and are supposed to cause distinct symptoms on leaves (Caballero *et al.*, 2009; van Lent *et al.*, 1980). Transmission can occur by vectors in a non-persistent manner (Brito *et al.*, 2012; Tavasoli *et al.*, 2009; Almeida *et al.*, 2005) and mechanically due to inoculation or grafting (Li *et al.*, 2013; Martin and Bristow, 1988). The economic impact of carlaviruses is demonstrably significant for agricultural important crops as potatoes (Li *et al.*, 2013; Massa *et al.*, 2006), garlic (Chen *et al.*, 2002), hop (Eastwell and Druffel, 2012) and ornamental plants as well (Singh *et al.*, 2012; Wylie and Jones, 2012; Caballero *et al.*, 2009; Eastwell *et al.*, 2009). The genus *Carlavirus* belongs to the *Betaflexiviridae* within the order Thymovirales and contains 47 members and tentative members respectively (ICTV, 2015), though the number is increasing. The genome of carlaviruses is organized as positive orientated monopartite single stranded (+)RNA with 8.3-8.7 kb in length and comprises six

open reading frames (ORF). A methylized cap or a monophosphate completes the 5'-terminus, the 3'-terminus is polyadenylated. Largest 5'-proximal ORF (ORF1) is processed monocistronicly into a 215-223 kD polyprotein. It comprises five conserved functional protein domains, including a viral methyltransferase (Vmethyltransf, pfam01160) near the 5'-terminus functioning in capping the viral RNA. A 2-oxoglutarate and Fe(II)-dependent oxygenase (2OG-FeII_Oxy_2, pfam13532) is located downstream, which includes AlkB-alkylated DNA repair protein. Subsequently, domains for a carlavirus endopeptidase (Peptidase_C23, pfam05379), a viral RNA helicase of superfamily 1 (Viral_helicase1, pfam01443) and a catalytic RNA-dependend RNA polymerase (RdRp2, pfam00978) complete ORF1 (Martelli *et al.*, 2007). Thus, it contains structures of the "Alpha-like" supergroup (Rozanov *et al.*, 1992). The following set of partially overlapping ORFs 2-4 code for three tripple gene block proteins (TGBp1-3). TGBp1 (ORF2, 25-26 kD) possesses ATPase, RNA-binding and RNA-helicase activities and is supposed to increase the size exclusion limit of plasmodesmata (Morozov and Solovyev, 2003). The product of ORF3 (11-12 kD) is a transmembrane protein belonging to plant viral movement protein family (Plant_vir_prot, pfam01307), which comprises movement proteins known from carlaviruses and other ssRNA plant viruses. Consecutively, ORF4 (7 kD) codes for a viral coat protein that belongs to the 7kD_coat superfamily (pfam02495) and is solely known from members of *Carla-* and *Potexvirus*. The three proteins are supposed to contribute concertedly to cell-to-cell movement of the viral nucleic acid molecules. ORF5 (32-36 kD) comprises two conserved domains coexisting in RNA-binding capsid proteins with zinc ribbon motif (Gramstat *et al.*, 1990). The conserved protein domain located at the 5'-proximal terminus of ORF5 belongs to Flexi_CP_N (pfam00286) superfamily and coexists with Flexi_CP (pfam08358) superfamily in capsid proteins of plant infecting carlaviruses. These three proteins and a cysteine-rich nucleic acid binding protein coded within ORF6 (11-16 kD) are transcribed as two monocistronic subgenomic mRNAs (Martelli *et al.*, 2007). Short non-coding regions (NCRs) are located at the 5'- and 3'-terminus (King *et al.*, 2012). Deviating from the genome organization scheme valid for the majority of classified carlaviruses, some members show extra features as having an CD for an ovarian tumor-like cysteine protease within ORF1 (Ho *et al.*, 2016) or lacking the cysteine-rich protein coded within ORF6 and replacing them with a protein of family atypical structure (De Souza *et al.*, 2013).

Within the genus *Carlavirus* the number of identified members has widely expanded for a few years as well as the number of host species ranging from crops to ornamental and woody plants (Ho *et al.*, 2016; Fujita *et al.*, 2015; Li *et al.*, 2013; Gutiérrez *et al.*, 2013; Eastwell and Druffel, 2012; Hatlestad *et al.*, 2011). The progress in carlavirus investigations is mainly driven by advances in sequencing methods. Classical clone based Sanger sequencing is now completed by next generation sequencing techniques that allow the identification of multiple viruses within one sample with manageable effort. With introduction of the new sequencing approaches, methodological bottlenecks occurring during the investigations of virus characteristics can be vanquished by revealing broad ranges of genomic sequence composition, which led to the discovering of a multitude of new viruses so far (Massart *et al.*, 2017; Prabha *et al.*, 2013). Virus identification is accelerated by *de novo* sequencing and uncovers the presence of new viruses and plant species not yet categorized as virus host respectively. In particular, woody plants like trees in forests and urban green are known to be affected by viruses (Büttner *et al.*, 2013). Viruses belonging to the genera *Ilarvirus*, *Nepovirus* and *Tombusvirus* have been frequently detected in *Ulmus* spec. for decades (Novák and Lanzová, 1980; Jones

and Mayo, 1973; Ford *et al.*, 1972; Schmelzer, 1969). Combining classical detection methods and NGS approaches increases the potential of virus identification. By expansion of applicable techniques so far underrepresented virus genera, such as *Carlavirus*, are spotlighted now.
European white elm trees (*Ulmus laevis* Pall.) in the park of Caputh located 30 km southwest of Berlin (Germany) were monitored regularly regarding virus-like symptoms, such as chlorotic ringspots, mottling, necroses and leaf deformation. Previous molecular and serological investigations revealed the presence of a flexuous particle in partially purified leaf extracts from elms with approximately 800 nm in size indicating a poty- or carlavirus as causing agent (Bandte *et al.*, 2004). An RT-PCR-based approach was conducted. Degenerated primers against regions of the genome of the genus *Carlavirus* proven to be suitable for detection of carlaviruses belonging to diverse species (Gaspar *et al.*, 2008; Nie *et al.*, 2008; Chen *et al.*, 2001; Badge *et al.*, 1996) were used for establishing an RT-PCR protocol. By this approach, carlaviruses couldn't be detected in any of the elm trees exhibiting symptoms (Eisold *et al.*, 2013). Nevertheless, the hypothesis of a virus infection wasn't rebutted and intense investigations were undertaken to confirm it. First remarkably results are reported here.

MATERIALS AND METHODS

European white elm trees (*Ulmus laevis* Pall.) in the park of Caputh expressing virus-like symptoms were monitored regularly from 2009 to 2014. Leaves of diseased and symptomless trees were sampled during each vegetation period monthly. From symptomatic leaves, areas with ringspots were cut and 70 mg fresh material was used for RNA extraction with Invitrap Spin Plant RNA Mini Kit (STRATEC Molecular) according to manufacturer's instruction. Residual DNA was removed with rDNase provided in NucleoSpin® RNA Kit (Macherey-Nagel) followed by cleaning the sample with NucleoSpin RNA clean-up Kit (Macherey-Nagel). To deplete plant abundant large ribosomal RNA molecules efficiently from total RNA isolated from elm leaves, the RiboMinus Plant Kit for RNA-Sequ (Invitrogen) was conducted with 10 µg of high integrity total RNA. Double-stranded full-length cDNA was synthesized with 1-2 µg RiboMinus RNA applying the Maxima H Minus Double-Stranded cDNA Synthesis Kit (Thermo Scientific) primed with random hexamers. For RNA sequence analysis approximately 1-2 µg of double-stranded cDNA were sent to BaseClear (Netherlands). Paired-end 100 bp sequence reads (≈ 50 Mb) were generated conducting Illumina Hi Seq2500 system. Reads were mapped and virus sequences *de novo* assembled on Biolinux and CLC Genomics Workbench respectively. Out of a dataset of 1,011,396 paired-end reads 908 contigs were constructed and used to identify viral sequences. Analysis with obtained sequences was performed by Clustal W (Larkin *et al.*, 2007) and Geneious version 9.1.3 (http://www.geneious.com, Kearse *et al.*, 2012). Sequences of representative *Carlavirus* species were obtained from online database and compared with contig sequences applying BLASTX 2.2.25 (Altschul *et al.*, 2007) in order to determine affiliation to predicted virus genera. Partial genomic sequences were deposited at EMBL under accession numbers LS999823, LT898349 and LT898350. Search for protein domains was conducted with Conserved Domain Database at NCBI (Marchler-Bauer *et al.*, 2009).

RESULTS AND DISCUSSION

As preliminary results pointed to a carlavirus as causal agent of the symptoms observed in elms (Bandte *et al.*, 2004) and RT-PCR diagnostic failed, alternatively Illumina high throughput sequencing was conducted. Three contigs were obtained supposed to cover broad ranges of the carlavirus genome, that vary in size ranging from 7739 nt to 8237 nt. Five out of six open reading frames (ORFs) characteristic for carlaviruses (Adams *et al.*, 2004, Figure 1) are coded within the sequences of the tentatively named *Elm carlavirus* isolate C3 (ElmCV-C3, 8112 nt in length, LT898349) and *Elm carlavirus* isolate C2 (ElmCV-C2, 8237 nt in length, LS999823). Sequence of *Elm carlvirus* isolate C17 (ElmCV-C17, 7739 nt in length, LT898350) features four ORF's.

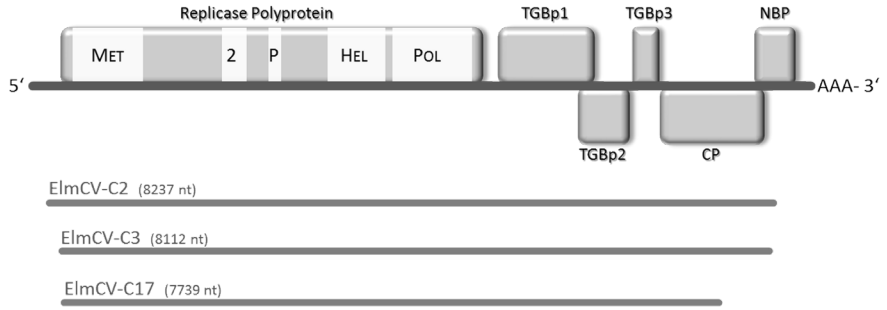


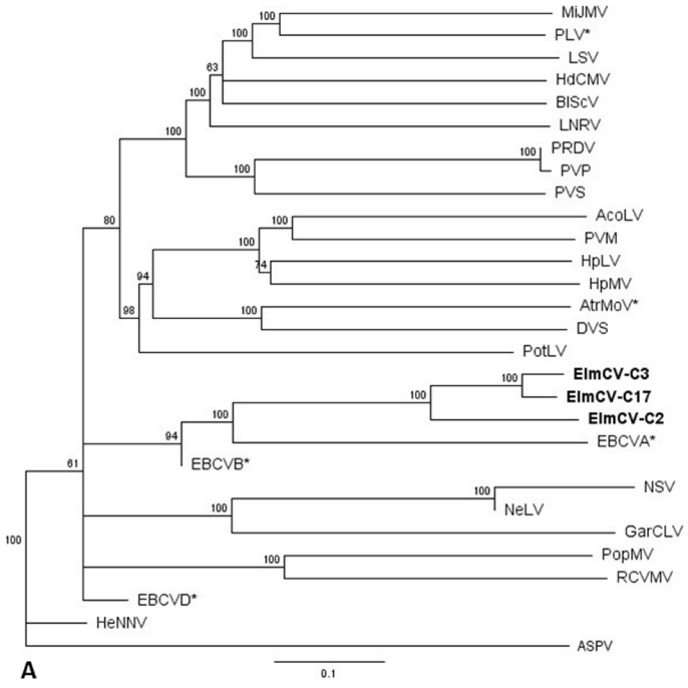
Figure 1: Carlavirus genome organisation. Blocks represent six open reading frames coding for viral replicase polyprotein, triple gene block proteins 1, 2, 3 (TGBp1, TGBp2, TGBp3), viral coat protein (CP) and nucleic binding protein (NBP). Protein domains for viral methyltransferase (Met), 2-oxoglutarate and Fe(II)-dependent oxygenase (2), carlavirus endopeptidase Peptidase_C23 (P), Viral_helicase1 (Hel) and RNA dependent RNA polymerase (Pol) within the replicase polyprotein are indicated by light grey. Bars show sequence overlays of ElmCV-C2, ElmCV-C3 and ElmCV-C17 with carlavirus genome.

The five ORF's detected within the three ElmCV sequences show high specific hits for protein superfamilies that are described to be present in carlavirus genomes (Table 1) (Ho *et al.*, 2016; Su *et al.*, 2015; Tang *et al.*, 2010). The first ORF of the ElmCV-C2, -C3 and -C17 isolate genomes codes for a polyprotein with five functional conserved protein domains including the viral methyltransferase, oxygenase, endopeptidase, helicase and the RdRp (Marchler-Bauer *et al.*, 2009). The protein structure of the viral methyltransferase scores a domain-specific e-value threshold of 1.78e-89 – 4.48e-92 giving high confidence to predicted CD affiliation. ORF2, ORF3 and ORF4 encode the triple gene block proteins (TGBp1, 2, 3). TGBp1 includes a viral helicase domain with a significant e-value for specific hit of 1.55e-42 – 3.79e-39. TGBp2 contains the viral movement protein domain Plant_vir_prot (e-values for specific hits 6.28e-30 – 1.94e-29) and together with TGBp3 a 7 kD viral coat protein (e-values for specific hit 2.12e-14 – 1.93e-13) they include conserved central domains common to carlaviruses. Two consecutive coat proteins are encoded by ORF5 (Flexi_CP_N with e-value for specific hits of 9.01e-20 – 9.20e-20 and Flexi_CP with e-values for non-specific hits of 7.77e-48 and 8.65e-48).

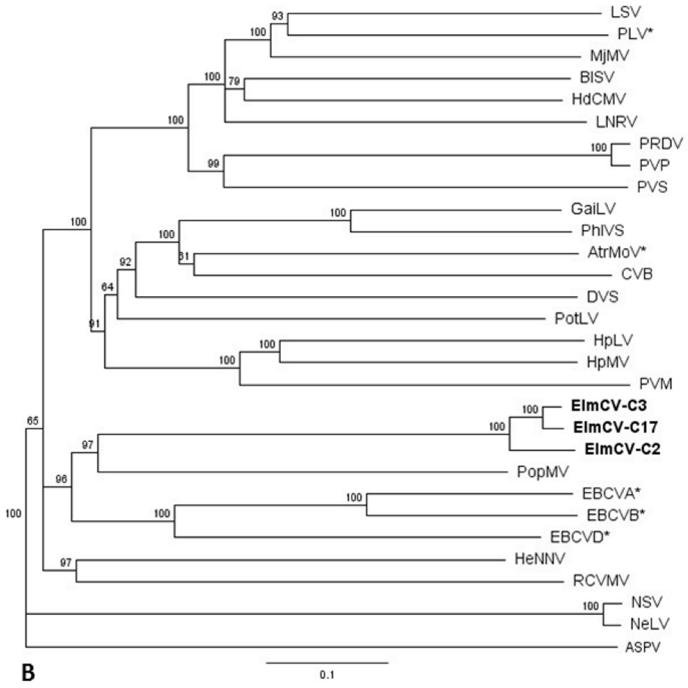
Table 1: Conserved domains (CD) detected within predicted open reading frames (ORF) of ElmCV-C2, -C3 and –C17 sequences. Protein sequences were analyzed using Conserved Domains searching tool of NCBI database (Marchler-Bauer *et al.*, 2009); tripple gene block protein (TGBp), coat protein (CP).

				ElmCV-C2		ElmCV-C3		ElmCV-C17	
ORF	**Protein and size [aa]**		**specific hit for CD**	**CD [aa]**	**E-value**	**CD [aa]**	**E-value**	**CD [aa]**	**E-value**
1	replicase polyprotein	1774	Vmethyltransf	1-308	4.48^{-92}	3-300	1.39^{-90}	3-300	1.78^{-89}
2	TGBp1	234	Viral_helicase1	25-226	1.55^{-42}	25-226	2.31^{-40}	25-226	3.79^{-39}
3	TGBp2	112	Plant_vir_prot	5-106	6.28^{-30}	5-112	1.17^{-29}	5-106	1.94^{-29}
4	TGBp3	60/71	7kD_coat	7-57	1.93^{-13}	7-68	2.12^{-14}	7-68	1.31^{-13}
5	CP	306	Flexi_CP_N	59-110	9.20^{-20}	59-110	9.01^{-20}		

By comparison with whole genomes of representative species belonging to the genus *Carlavirus*, the ElmCV isolates show overall nucleotide sequence identities of 40.1–74.0 %. Highest identities were found for EBCVA and EBCVD matching in 74.0 % and 71.0 % with ElmCV-C3 and matching in 73.8 % and 70.4 % with ElmCV-C17. With ElmCV-C2, the sequence identities of 69.2 % and 71.9 % are similar to the general range of identities with other members of the genus *Carlavirus*. Basically, within the RdRp motif matches were found that show accordance between 66.7 % and 73.8 % with all compared carlavirus genomes. In particular, identities of 73.8 % and 73.4 % between EBCVA and the sequences of ElmCV-C2 and ElmCV-C3 as well as 71.2 % identity of ElmCV-C3 with EBCVB within the RdRp motif indicate close relationships. The ElmCV isolates show nucleotide sequence identities of 79.5–93.6 % to each other, which corresponds with 90.4 % and 96.8 % on peptide level for the replicase polyprotein and 99.0 % identity for the coat protein. To prove a close relationship of ElmCV-C2, -C3 and -C17 with representative members of *Carlavirus*, a phylogenetic tree was constructed using the neighbor-joining algorithm. Based on the whole genome comparison, ElmCV-C2, -C3 and -C17 were grouped together with EBCVA and EBCVB. On amino acid level of ORF1, ElmCV isolates cluster closely together with PopMV and EBCVA forms a distinct but closely related clade together with EBCVB (Figure 2). Identity values range from 56.4–55.8 % between ElmCV isolates and EBCVA and 71.9–74.0 % between ElmCV isolates and EBCVB. In contrast, the polypeptides of PopMV and ElmCV-C2, -C3 and -C17 share an identity of 47.1–47.4 %. Comparison at the amino acid level of the coat protein of ElmCV-C2, -C3 and -C17 with other carlaviruses revealed similarities between 22.1 % and 60.8 %.


MiJMV
PLV*
LSV
HdCMV
BlScV
LNRV
PRDV
PVP
PVS
AcoLV
PVM
HpLV
HpMV
AtrMoV*
DVS
PotLV
ElmCV-C3
ElmCV-C17
ElmCV-C2
EBCVA*
EBCVB*
NSV
NeLV
GarCLV
PopMV
RCVMV
EBCVD*
HeNNV
ASPV
0.1

A


LSV
PLV*
MjMV
BlSV
HdCMV
LNRV
PRDV
PVP
PVS
GaiLV
PhlVS
AtrMoV*
CVB
DVS
PotLV
HpLV
HpMV
PVM
ElmCV-C3
ElmCV-C17
ElmCV-C2
PopMV
EBCVA*
EBCVB*
EBCVD*
HeNNV
RCVMV
NSV
NeLV
ASPV
0.1

B

Figure 2: Phylogenetic relationship of ElmCV isolates from Germany generated using the Jukes-Cantor genetic distance model of the whole genome (A) and the replicase polyprotein (B) compared to representative members of the genus *Carlavirus* with *Apple stem pitting virus* (ASPV - genus *Foveavirus*, KF321967) as outgroup. Still unclassified carlaviruses are labelled with asterisks. The unrooted trees were generated using the neighbour-joining algorithm of Geneious 9.1.3 software. Bootstrap values (1000 bootstrap replicates) are shown at the branching points when >60 %. The scale bar represents the number of substitutions per position. *Aconitum latent virus* (AcoLV, AB051848.1), *Atractylodes mottle virus* (AtrMoV, KR349343.1), *Blueberry scorch virus* (BlScV, AY941198.1), *Chrysanthemum virus B* (CVB, AB245142.1), *Daphne virus S* (DVS, AB889483.1), *Elderberry carlavirus A* (EBCVA, KJ572560.1), *Elderberry calravirus B* (EBCVB, KJ572561.2), *Elderberry carlavirus D* (EBCVD, KJ572563.1), *Gaillardia latent virus* (GaiLV, KJ415259.1), *Garlic common latent virus* (GarCLV, JQ899445.1), *Helleborus net necrosis virus* (HeNNV, AB623047.1), *Hop latent virus* (HpLV, AB032469.1), *Hop mosaic virus* (HpMV, EU527979.1), *Hydrangea chlorotic mottle virus* (HdCMV, EU7547202), *Ligustrum necrotic ringspot virus* (LNRV, EU074853.1), *Lily symptomless virus* (LSV, AM263208.1), *Mirabellis jalapa mottle virus* (MiJMV, JN039374.1), *Narcissus symptomless virus* (NSV, AM182569.2), *Nerine latent virus* (NeLV, JQ395044.1), *Phlox virus S* (PhlVS, EF492068.1), *Poplar mosaic virus* (PopMV, AY505475.1), *Potato latent virus* (PotLV, EU433397.2), *Potato rough dwarf virus* (PRDV, EU020009.1), *Potato virus P* (PVP, EU338239.1), *Potato virus S* (PVS, LN851190.1), *Red clover vein mosaic virus* (ACN58188.1).

The data indicate consistently ElmCV to be a new member within the genus *Carlavirus* according to King *et al*. (2012), by which distinct *Carlavirus* species share less than 72 % on nucleotide or 80 % on amino acid level in RdRP and CP genes respectively. The demarcation of ElmCV-C2, -C3 and -C17 to each other basing on sequence analysis is supposed to be below species distinction level. Most likely, ElmCV-C2, -C3 and -C17 are different isolates of the *Elm carlavirus*. Completing the ElmCV genome that still misses the 5'-terminal NCR, the 3'-terminal sequence part of ORF5 in ElmCV-C17 genome, ORF6 and the 3'-terminal NCR with poly-A tail, will close remaining gaps within the molecular characterization. Therewith, the whole genome organization of ElmCV can be determined. To receive all-encompassing data about plant pathogenic viruses, further properties, such as serological relationship, distribution, transmission and host range, have to be taken into consideration. The verification of an association of ElmCV with observed virus-like symptoms will succeed by transmission tests fulfilling Koch's postulates. This study provides first information about molecular structure and organization of a new *Carlavirus* detected in *U. laevis* in Germany.

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